"A Unified Metabolism for Mars--A New Approach Developed from Research on Extraterrestrial Life Detection by Enzymatically Induced Exchange of ^{18}O"

FINAL REPORT

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During the past year we have continued the development of a general "graded" life detection approach ranging from environmental and *in situ* observations to specific metabolic experiments. In addition, we have attempted to further expand the scope of available biologically-oriented measurements, placing particular emphasis on the development of soil analyses which are compatible with our technical constraints.

We have also made a good deal of progress in solving some of the technical aspects of this program. The development and construction of a zero-dead-volume leak and the computer-mediated data acquisition system, in particular, greatly increased the scope of our experimental capability.

I. The Exchange of Oxygen between Sulfate and Water*

During the past year, we reopened a study on the inorganic exchange of oxygen from sulfate to water which was initiated several years ago. (See Annual Report for NASW-1735) A fundamental requirement of any anion used in assays involving the transfer of oxygen atoms is that it have a negligible exchange rate with water under the conditions used for the bioassay. E. G. Behrman (Ohio State) reopened the question of inorganic $SO_4^=$ - H_2O oxygen exchange in a personal communication, suggesting that sulfate might undergo nucleophilic attack by water and/or OH^-. We consequently have re-examined this question.

Our investigation suggested that, using H_2O as a nucleophile, the second order rate constant $k_2 < 3.3 \times 10^{-12}\ M^{-1}\ sec^{-1}$. This corresponds to a half time $t_2 > 120$ years at 100°C. Similar experiments also showed that, using OH^- as a nucleophile, the second order rate constant $k_2 < 2 \times 10^{-10}\ M^{-1}\ sec^{-1}$. By way of comparison, we found $k_2 = 5.3 \times 10^{-5}\ M^{-1}\ sec^{-1}$ for the acid-catalyzed exchange reaction at 100°, a value in good agreement with data available in the literature (J. C. Hoering and J. W. Kennedy, J. Amer. Chem. Soc. 79, 56 (1957)). It thus appears that sulfate oxygen does not undergo appreciable exchange with the oxygen of H_2O by any mechanism other than acid-catalyzed dehydration under these conditions.

* A detailed description of this work appeared in Inorganic Chemistry 11, 1162 (1972).

II. Phosphate-H_2O Exchange Experiment

During the past year, many experiments have been performed in which the exchange of ^{18}O between phosphate and water is monitored by the appearance of ^{18}O in the CO_2 present in the reaction vessel. Only rarely has this experiment attained a biological sensitivity which is comparable to that obtained in the experiment from which it is derived (Kok and Varner, Science 155, 1110 (1967)*. These discrepancies could be due to the use of soils which might, for example, cause the precipitation of the added $P^{18}O_4$ by heavy metals or the dilution of the added label phosphate with unlabeled phosphate present in the soil. Alternatively, the modified method may be compromised by the presence of factors which inhibit the CO_2-H_2O oxygen exchange reaction and/or the appearance of other species at masses 44-46.

Experiments performed to determine whether added phosphate-^{18}O might be precipitated or otherwise sequestered in the soil demonstrated that at least 60% of the added phosphate could be recovered by extraction with H_2O. Similar experiments also showed that there is no appreciable water-extractable phosphate (or silicate) in this soil. This suggested that a) there was no significant dilution of the labeled phosphate

* In the experimental method described by Kok and Varner, the H_2O is recovered from the soil by distillation and analyzed for ^{18}O content. In the variant currently being tested, the ^{18}O content of the H_2O is deduced from the ^{18}O content of the CO_2 present in the vessel.

by unlabeled phosphate and b) the experiment which was done to determine the recovery of the phosphate from soil was not confounded by the presence of silicates. (Silicates can interfere with the Fiske-SubbaRow phosphate determination used in these experiments.) Thus, it appears that the poor results of the experiment cannot be attributed to the loss or dilution of labeled phosphate.

Because ^{18}O is such a "mobile" isotope, we also performed a study to determine the degree to which metabolic exchange kinetics might be confounded by non-biological exchange of the ^{18}O label. This type of reaction would be particularly significant in cases in which the soil contained large quantities of silicates.

Measurements using the mass spectrometer and ^{18}O labeled water have shown that it is very difficult to predict the amount of available exchangeable oxygen present in a sample. For example, in one experiment in which there was a large excess of silicate, the signal was degraded only 30% after six days, suggesting that only a few percent of the oxygen in the sand was available for exchange.

A computer study was also initiated to study some of the "worst cases;" that is, cases in which the exchangeable oxygen in the soil is much greater than the amount of ^{18}O label originally added. By using rate constants for these exchange reactions available in literature and/or determined in our laboratory, we were able to study some of the kinetic aspects of these "worst cases." For example, in cases in which the exchange of oxygen is slower with the "soil exchangeable oxygen" than with the CO_2 used to monitor the isotope in the aqueous phase, one would

expect to see a "transient," provided that the rate of $H_2{}^{18}O$ production is comparable to the rate of exchange into silicate. These numbers were then translated into metabolic rates required to get a measurable signal in the presence of different amounts and types of exchangeable oxygen. In this way we were able to determine the theoretical sensitivity of these assays under these particular compromising conditions. These studies suggested that the kinetics of the H_2O-CO_2 exchange reaction should not confound the results and interpretation of the ^{18}O exchange experiment under the conditions employed.

In an effort to determine whether the sensitivity of the $P^{18}O_4$-H_2O exchange experiment might be affected by the appearance of species other than CO_2 at masses 44 thru 46, the experiment was performed by two different methods: a) a method in which the CO_2 $^{18}O/^{16}O$ ratio was determined in the experimental vessel; and b) a method described previously (Annual Report, May 15, 1969, NASw 1735), in which the water was distilled off and equilibrated with CO_2 in a separate vessel. These experiments showed that there was, indeed, a significant difference between the CO_2 oxygen isotope ratio measured in the vessel and the ratio measured in CO_2 equilibrated with H_2O which was recovered from the soil by distillation. Thus, the most likely hypothesis for the poor results obtained in the PO_4-H_2O exchange experiments is that another specie appears at mass 44, thereby suppressing the measurable 46/44 isotope ratio. Although it appears that the modified PO_4-H_2O oxygen exchange experiment as currently conceived may still be feasible, it is not a particularly sensitive life detection assay and, compared to some of the other bioassays we have described, appears to be a low priority experiment.

III. Temperature-Programmed In Situ Experiment

During the past year, a good deal of effort has been expended on results related to a bioassay which we call a temperature-programmed in situ. This assay is an outgrowth of the in situ control and the desire to increase the amount of information obtained from in situ incubations. The basis of the bioassay is the use of a temperature regime to analyze two soil samples, one before and one after incubation. Any differences between the released volatiles (and possibly pyrolysis products) of the two samples would be an indication of biological activity.

The experiments that we have done to date suggest that we are on the right track. However, these studies have been severely hampered by a massive data-handling problem. Because of these difficulties we have been unable to determine the biological feasibility of this experiment, although we have learned a good deal relating to its technical implementation. We have therefore acquired a PDP-8 minicomputer as an on-line data reduction system for this experiment (see below). We have suspended work on the biological aspects of this experiment until all facets of this data-handling system are in operation.

IV. The Non-biological Gas Exchange of Soils

One of the most important phenomena which limits the proposed biological experiments is the non-biological gas exchange of soils due to external perturbations. These adsorbtion-desorbtion phenomena will have a particularly significant impact on the *in situ* experiment, since in this experiment we may be limited as much by background reactions as by technical performance.

During the early phases of this contract many experiments were performed in an attempt to determine the effect of partial pressure changes on the adsorbtion-desorbtion reactions of soil. During the course of these studies (and those related to the detection of trace organic compounds (see below)) we found that our method of gas handling could significantly affect the composition of the gas phase. Consequently, these studies were suspended pending the development of a zero-dead volume inlet valve (see below).

Although the data we acquired were compromised due to the aforementioned gas handling difficulties, these experiments did suggest that under the conditions tested adsorbtion would not profoundly affect the validity of the biological measurements. We plan to repeat these experiments using our recently improved gas handling system to obtain a more precise determination of any of these adsorbtion effects.

V. Factors Affecting the Detection of Trace Organic Compounds

One of the strongest arguments for the presence of "life" would be the observation of newly-formed organic compounds after the incubation of a soil sample. We have therefore attempted to determine some of the parameters which limit the detection of these compounds.

During the early phases of this project we performed many experiments in an attempt to determine the minimum detectable quantity of organic compounds of biological interest, such as formic, acetic and propionic acids, butanol, ethanol, etc. under incubation conditions. However, we found that the method of sampling could have a profound effect on the sensitivity of the mass spectrometer for the detection of organic compounds present in the vapor phase. In particular, the adsorption of gases and volatiles by the sample line significantly affected the attainable sensitivity.

After the design and fabrication of a suitable gas handling system (see below) we repeated and extended this series of experiments. We found that: 1) the vapor pressure, and consequently, the minimum detectable quantity, of an organic compound was not strongly affected by its milieu (sand, humus, etc.); and 2) the vapor pressure of organic compounds at very low concentrations deviated markedly from that predicted by Raoult's law, (i.e., $P=XP^{o}$, where P is the partial vapor pressure above a solution, P^{o} the vapor pressure of the pure liquid,

and X its mole fraction), or Henry's law, (i.e., $P = kX$, where P and X are the same as in Raoult's law, and k ($\neq P^{o}$) a constant.) We recently obtained a publication from the National Bureau of Standards ("Thermodynamic Properties of Compounds of Biochemical Interest in Aqueous Solution") which may greatly enhance an understanding of some of the important factors related to this problem.

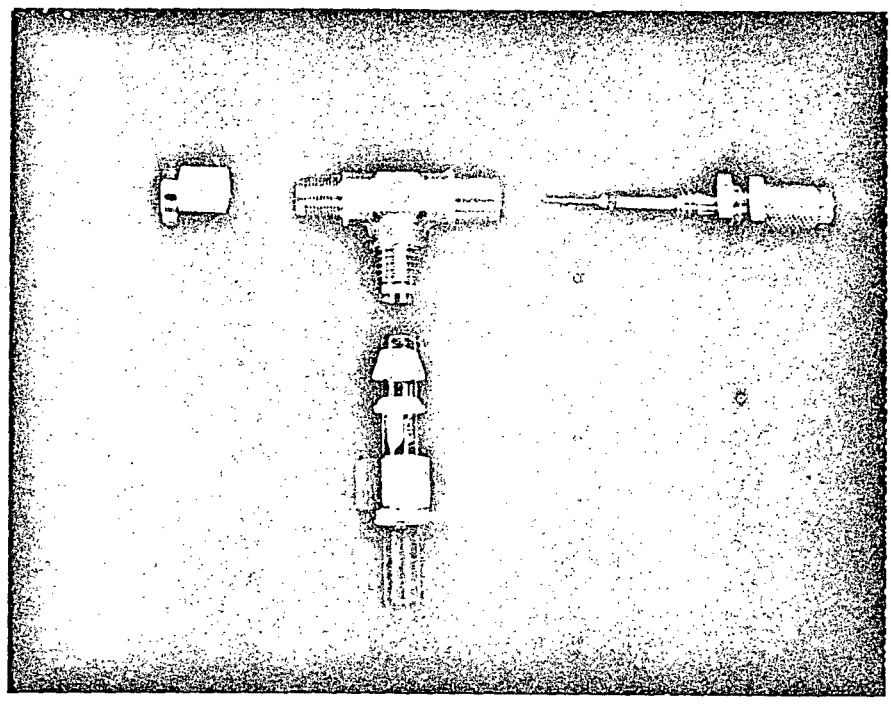
VI. Sample Inlet System

Many experiments which we have attempted to perform during the early phases of this contract were stymied by the lack of a suitable soil sample-gas handling system. The principal disadvantages of the system being used were: A) each measurement required ca. 5 ml of gas (at ~ 1 atmosphere pressure) which in turn required that the soil sample be large; and B) in some cases the gas sampling system perturbed the composition of the gas being measured due to the adsorbtion of species by the evacuated sample volume walls. These problems were due largely to the presence of a sample line volume between the leak valve on the mass spectrometer and the sealing point (valve) on the sample vessel when it is attached to the mass spectrometer.

We have recently designed and constructed a soil ampoule-gas handling system which circumvents these problems. In the new design, a series of sample vessels which contain both the sealing point and leak at the same position are connected to a manifold which feeds directly into the mass spectrometer and is kept at mass spectrometer pressure. The heart of this assembly is a Nupro SS-4AS needle valve which, although designed for metering, can be closed completely if caution is exercised. (In critical experiments we plan to use gold-plated valves). This valve is connected to the mass spectrometer

and the sample vessel using Swagelok fittings with Teflon ferrules. The sample vessel itself is a Kimax 2806 test tube (6 x 50 mm, total volume ~ 0.8 ml). Exploded and assembled views of this apparatus are shown in Figure 1. These valves can then be attached directly to the high-vacuum mass spectrometer inlet by a manifold as illustrated in Figure 2.

Because this system has no volume of sample line which is alternately filled and evacuated, the composition of the gas phase entering the mass spectrometer will more accurately reflect the gas phase above the soil sample, since the sample gas will not be altered due to adsorbtion on the walls of the sample inlet. In addition, much smaller gas volumes will be required for analysis. In the old method, ca. 5 ml of gas at ampoule pressure was required; the new system requires ca. .05 μl/sec.



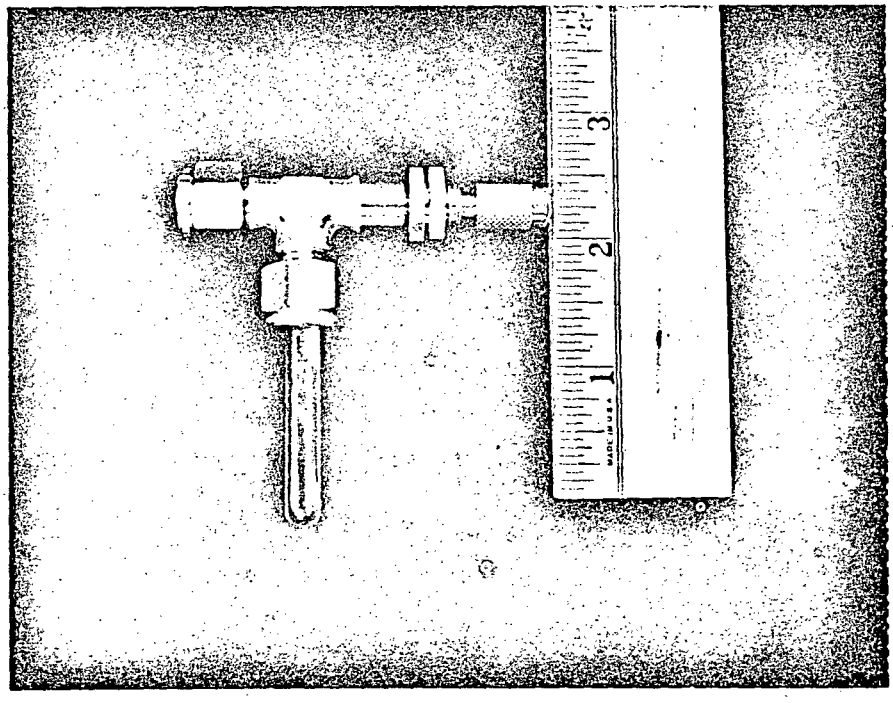


Figure 1. Exploded view (top) and assembled view (bottom) of leak valve-sample ampoule module.

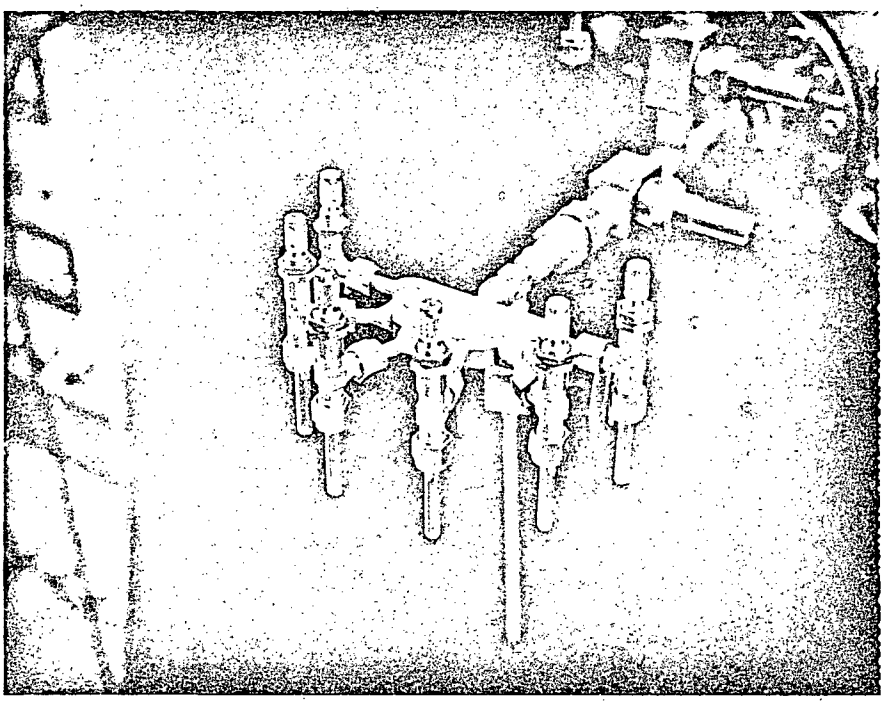


Figure 2. Sample manifold with attached leak valve-sample ampoule modules.

VII. Computer-Mediated Mass Spectrometer Data - Handling System

During the past year we completed the design and development of hardware and software for a computer-mediated mass spectrometer readout system. This system allows us to acquire averaged mass spectrometer data over the entire dynamic range of the mass spectrometer (~ 6 orders of magnitude). The system reads and stores the background and sample spectra, subtracts the background from the sample, and normalizes the amplitude at each mass number to the amplitude at mass 40 (Argon). Both sample and background are currently analyzed at six attenuations with five repetitions at each attenuation to provide some signal averaging. With minor modifications, this apparatus will also allow us to process and analyze the massive amount of data acquired during the temperature programmed in situ experiments described above.

The heart of this data-handling system is a PDP-8E minicomputer. This computer has a fixed 12-bit word length with 8000 words of core memory. Input devices to the machine consist of a teletype (ASR-33) and a 10-bit A/D converter.

The algorithm used during the course of a scan is as follows: After receiving an initial starting pulse, the scan voltage and the signal

amplitude from the mass spectrometer are measured and digitized by the 10-bit A to D converter. By the use of a tabular look-up routine, the mass spectrometer then determines whether the measured scan voltage corresponds to "peak" or "valley" in the spectrum. If the position does correspond to a peak, the mass number is then determined in a similar manner. The computer then stores the maximum signal amplitude obtained in each peak "window" in a location corresponding to its mass number.

After each scan, the attenuators on the mass spectrometer output are automatically switched and the spectrum is again processed as described above. We are currently using a system in which six attenuations are sequentially scanned five times each, for a total of 30 scans. At the end of these 30 scans, the acquired data are scaled, averaged and stored as floating point numbers.

After both the background and sample spectra have been acquired and processed in this manner, the data are presented in the following format: for each mass number, the background amplitude, the sample amplitude, and "(sample minus background)/ Argon 40" (normalized signal), are printed out on the teletype.

VIII. Soil Analysis

During the past year we attended several meetings and conferences on Viking and post-Viking experiments. We came away with the impression that a biological experiment slanted to or combined with soil analysis might be the preferred approach to later Viking missions from both a scientific and political viewpoint. Consequently, we have initiated a program to study and develop biologically oriented soil assays which will be compatible with our mass spectrometer-ampoule configuration.

Because of the current interest in the scientific community concerning the status of nitrogen on Mars, a good deal of our work on this program has been devoted to the detection and measurement of various nitrogen compounds in the soil. In this way it may be possible to determine the nitrogen balance on Mars.

For example, we have examined and tested the use of sulfamic acid as a reducing agent for the determination of nitrite and nitrate in soils. In a mildly acidic solution of sulfamic acid nitrite is reduced at room temperature to N_2, which can be measured mass spectrometrically.

Nitrate can be determined in a similar manner except that a concentrated acid solution is used. From the measurement of N_2O (M/E=44) the amount of NO_3^- can be determined. (To some extent, the above reaction will also convert the more accessible-NH_2 nitrogen into N). This overall procedure will thus yield the total amount of nitrite and nitrate present in the soil sample.

We have also studied the conversion of soil ammonia into molecular nitrogen by oxidation with hypobromite. In these experiments soil samples were reacted with a solution of sodium hypobromite for 30 minutes at 70°: The released N_2 was then measured mass spectrometrically.

We tested a modification Van Slyke method to determine the amount of amino nitrogen present in the soil. Experiments to date have been quite promising although we have encountered some difficulty in coping with the large amounts of NO released during the course of the reaction.

In addition to the soil assays related to the status of nitrogen on Mars, we have tested some of the more obvious soil parameters such as the acid release of CO_2 and SO_2 from carbonate and sulfites, the determination of total organic carbon by wet oxidation, etc.

IX. Parametric Studies of Bioassays

With the development of the small sample handling system described above, we were in a position to do parametric studies using some of the more "precious" soil samples received from Ames. In addition, we could perform many experiments using expensive isotopes at a great saving in cost. The use of the computer mediated mass spectrometer readout system greatly facilitated this work.

We have performed most of the "standard" bioassays (in situ, addition of yeast extracts, ^{13}C, $^{15}NO_3^-$, H_2 - D_2) on the six test soils obtained from Ames. In addition, ^{13}C - acetylene recently became available at a reasonable cost, and we thus were able to perform some soil nitrogenase assays (i.e. $C_2H_2 \xrightarrow{N_{2ase}} C_2H_4$) with greatly improved sensitivity.